United States Department of Agriculture
Center for Veterinary Biologics
Testing Protocol

SAM 203

Supplemental Assay Method for Potency Testing
Clostridium perfringens Type D Epsilon Antigen

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Supplemental Assay Method for Potency Testing *Clostridium perfringens* Type D Epsilon Antigen

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Supplemental Assay Method for Potency Testing *Clostridium perfringens* Type D Epsilon Antigen

1. Introduction

This Supplemental Assay Method (SAM) describes the method used to determine whether biological products containing *Clostridium perfringens* type D epsilon antigen can stimulate the production of satisfactory immunity as prescribed by the Code of Federal Regulations, Title 9 (9 CFR), Part 113.112. For products that require 2 vaccinations, rabbits are vaccinated twice 20-23 days apart and bled 14-17 days following the second vaccination. For products that require a single vaccination, rabbits are vaccinated and bled 34-40 days later. The serum is titrated by a toxin-antitoxin neutralization test using mice as an indicator.

2. Materials

2.1 Equipment

2.1.1 Mixer, vortex-type

2.1.2 Centrifuge

2.1.3 Autoclave

2.1.4 Freezers, -20°C and -70°C

2.1.5 Refrigerator, 2°-7°C

2.1.6 Micropipettes, 100-µl and 1000-µl

2.2 Reagents/supplies

2.2.1 *C. perfringens* type D epsilon antitoxin, IRP 249

2.2.2 *C. perfringens* type D epsilon toxin, IRP 450

2.2.3 Peptone diluent

2.2.4 Screw-top Erlenmeyer flask, 500-ml, with cap

2.2.5 Syringes, needle-locking, 1-cc, 10-cc, or 30-cc

2.2.6 Needles, 25- to 27-gauge x 1- to 1 1/4-inch, 20-gauge x 1-inch

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2.2.7 Vacutainer® needles, 20-gauge x 1 1/2-inch

2.2.8 Serum separation tubes, 12.5-ml

2.2.9 Pipettes, 5-ml, 10-ml, 25-ml

2.2.10 Tips for micropipettes

2.2.11 Ketamine hydrochloride, 100 mg/ml solution

2.2.12 Xylazine, 20 mg/ml solution

2.2.13 Water, distilled or deionized, or water of equivalent purity

2.2.14 Glass dilution bottles, 160-ml

2.2.15 Glass screw-cap tubes, 13 x 100-mm

2.2.16 Polystyrene snap-top tubes, 17 x 100-mm with caps

2.2.17 Polystyrene screw-cap tubes, 17 x 120-mm

2.2.18 Polypropylene conical screw-cap tubes, 50-ml

2.3 Test animals

2.3.1 New Zealand White rabbits, nonpregnant females, 4-8 lb (Eight rabbits are required per serial to be tested.)

2.3.2 White Swiss nonpregnant female mice, 16-20 g (Five mice are required for each toxin-antitoxin mixture.)

3. Preparation for the test

3.1 Personnel qualifications/training

Technical personnel need a working knowledge of the use of general laboratory chemicals, equipment, and glassware and must have specific training and experience in the safe handling of clostridial toxins. Personnel must have specific training in the care and handling of laboratory rabbits and mice.

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3.2 Preparation of equipment and supplies

3.2.1 Sterilize all glassware before use.

3.2.2 Use only sterile supplies (pipettes, syringes, needles, etc.)

3.2.3 Operate all equipment according to the manufacturers' instructions.

3.3 Preparation of reagents

3.3.1 Peptone diluent

Peptone (Difco)	8 g
NaCl, reagent grade	2 g
Water q.s. to	800 ml

Dissolve peptone and sodium chloride in water. Adjust pH to 7.2 with 1N sodium hydroxide. Fill a 500-ml Erlenmeyer flask no more than 3/4 full with diluent. Autoclave with caps loosened at 121°C for 25-30 minutes. Cool flasks and tighten caps. Store at 2°-7°C for up to 3 months.

3.3.2 Preparation of *C. perfringens* type D standard epsilon antitoxin

1. *C. perfringens* type D epsilon antitoxin, IRP 249, contains 50 antitoxin units per ml (AU/ml) and has been standardized against the World Health Organization *C. perfringens* (*C. welchii*) type D international antitoxin. Each vial contains 3.4 ml of antitoxin.

2. Prepare a dilution of antitoxin that contains 1.0 AU epsilon antitoxin per ml by adding 2.0 ml of IRP 249 to 98.0 ml of peptone diluent in a 160-ml glass dilution bottle. Dispense the diluted antitoxin in 2.5 ml amounts in 13 x 100-mm screw-cap tubes and store at -70°± 5°C until used. For the purpose of this test, the 1.0 AU/ml dilution of antitoxin is referred to as the standard epsilon antitoxin.

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3.3.3 Preparation of *C. perfringens* type D epsilon toxin

Prepare a 1:32 dilution of *C. perfringens* type D epsilon toxin by adding 1.0 ml of IRP 450 to 31.0 ml of peptone diluent in a 50-ml screw-cap tube. Dispense diluted toxin in 1.5 ml amounts in 13 x 100-mm screw-cap tubes. IRP 450, diluted 1:32, is stable when stored at -70°± 5°C.

4. Performance of the test

4.1 Vaccination of rabbits

4.1.1 Thoroughly shake each bottle of product and wipe the top with alcohol before filling the syringe.

4.1.2 Vaccinate each rabbit subcutaneously in the shoulder region with half of the largest recommended dose for any species indicated on the product label. Use 10-, 20-, or 30-cc syringes fitted with 20-gauge x 1-inch needles to vaccinate the rabbits.

4.1.3 For products that require 2 vaccinations, give the second vaccination 20-23 days after the first.

4.2 Collection and preparation of rabbit serum

4.2.1 Collect blood from the test rabbits 34-40 days after vaccination (or 14-17 days after the second vaccination for products that require 2 vaccinations).

4.2.2 Anesthetize rabbits for bleeding with a mixture of 1.32 mg/kg of xylazine and 8.8 mg/kg of ketamine hydrochloride. Give the anesthetic mixture by intramuscular injection.

4.2.3 Use a 12.5-ml serum separation tube fitted with a 20-gauge x 1 1/2-inch Vacutainer® needle to collect blood from the heart. Collect approximately 12.5 ml of blood from each rabbit. Gently invert tubes 5 times. Let the tubes of blood sit at 22°-26°C (room temperature) for 30-60 minutes.

4.2.4 Centrifuge blood at 1000 x g for 10-20 minutes at room temperature.

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4.3 Preparation of serum pools

4.3.1 Prepare a pooled sample using an equal volume of serum from at least 7 rabbits per vaccinated group (provided that, if more than 7 rabbits are bled per vaccinated group, then equal volumes from each rabbit are used for the serum pool). If less than 7 rabbits are bled, the test is invalid and should be repeated.

4.3.2 The pooled sample may be held at 2°-7°C for up to 7 days. If testing will not be completed within 7 days, store the pooled sample at -20°C or lower.

4.3.3 Use 1.0 ml pooled serum diluted with 1.0 ml peptone diluent to test for 2 AU/ml of antitoxin.

4.3.4 Use 1.0 ml pooled serum diluted with 2.0 ml peptone diluent to test for 3 AU/ml of antitoxin.

4.4 Toxin neutralization

4.4.1 Preparation of standard epsilon toxin

1. Further dilute the toxin prepared in **Section 3.3.3** to 1:320 by adding 1.0 ml of diluted (1:32) toxin to 9.0 ml of peptone diluent in a 17 x 100-mm snap-top tube. For the purpose of this test, the 1:320 dilution of IRP 450 is referred to as the standard epsilon toxin.

2. A volume of 0.6 ml standard epsilon toxin and 0.4 ml peptone diluent represents 10 L_o doses. A volume of 0.8 ml standard epsilon toxin and 0.2 ml peptone diluent represents 10 L_+ doses.

3. For the purposes of this SAM, 10 L_o dose is defined as the greatest amount of toxin that, when mixed with 1.0 AU, results in 100% survival of all mice inoculated intravenously (IV) with 0.2 ml of this mixture. The 10 L_+ dose is defined as the least amount of toxin that, when mixed with 1.0 AU, results in the death of 80%-100% of all mice inoculated IV with 0.2 ml of this mixture.

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4. Mix a sufficient volume of standard epsilon toxin and peptone diluent (0.6 ml of standard epsilon toxin and 0.4 ml of peptone diluent [10 L_o doses] in a 17 x 120-mm conical tube for each serum pool and the L_o control).

4.4.2 Preparation of standard epsilon antitoxin

Thaw *C. perfringens* type D epsilon antitoxin IRP 249, previously diluted 1:50 (see **Section 3.3.2**). This dilution contains 1 AU/ml and is referred to as the standard epsilon antitoxin.

4.4.3 Product and standard epsilon toxin

1. Add 1 ml of each of the serum dilutions (see **Section 4.3**) to 1 ml of this standard epsilon toxin-peptone diluent mixture (see **Section 4.4.1.4**) in 17 x 100-mm snap-top tubes. Mix each tube well using a vortex-type mixer.

2. Let the mixtures sit at 22°-26°C (room temperature) for 1 hour.

3. Place tubes in ice.

4.4.4 Standard epsilon toxin and antitoxin controls

1. Add 1.0 ml of standard epsilon antitoxin containing 1.0 AU/ml (see **Section 4.4.2**) to 1 ml of the standard epsilon toxin-peptone diluent (10 L_o doses) mixture (see **Section 4.4.1.4**) in a 17 x 100-mm snap-top tube. Mix well with a vortex-type mixer.

2. Add 1.0 ml of standard epsilon antitoxin containing 1.0 AU/ml (see **Section 4.4.2**) to a mixture containing 0.2 ml of peptone diluent and 0.8 ml of standard epsilon toxin (10 L_+ doses) in a 17 x 100-mm snap-top tube. Mix well with a vortex-type mixer.

3. Let the mixtures stand at 22°-26°C (room temperature) for 1 hour.

4. Place tubes in ice.

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4.5 Inoculation of mice

4.5.1 Inject 0.2 ml of each standard epsilon toxin-product antitoxin mixture into each of 5 mice.

4.5.2 Inject 0.2 ml of each standard epsilon toxin-standard epsilon antitoxin mixture into each of 5 mice.

4.5.3 Inoculate all mice into a lateral tail vein. Use 1-cc syringes fitted with 25- to 27-gauge x 1- to 1 1/4-inch needles.

4.5.4 Always inoculate the mice receiving the standard epsilon toxin-standard epsilon antitoxin mixtures (controls) **last**.

4.5.5 Mouse inoculations should be completed within 1 hour of placing the toxin-antitoxin mixtures in the ice.

4.5.6 The test is concluded 24 hours after the mice are inoculated.

5. Interpretation of test results

5.1 Criteria for a valid test

5.1.1 All 5 mice inoculated with the standard 10 L_o/1.0 AU control mixture must survive.

5.1.2 At least 4 of the 5 mice inoculated with the standard 10 L_+/1.0 AU control mixture must die.

Note: Moribund animals exhibiting clinical signs consistent with the expected disease pathogenesis that are unable to rise or move under their own power may be humanely euthanized and considered as deaths as outlined in 9 CFR 117.4.

5.2 Interpretation of test results

5.2.1 If 5 of the 5 mice inoculated with the 1:2 diluted pooled serum-standard epsilon toxin mixture survive, the serum contains at least 2 AU/ml of

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C. perfringens epsilon antitoxin, and the product is satisfactory.

5.2.2 If 5 of the 5 mice inoculated with the 1:3 diluted pooled serum-standard epsilon toxin mixture survive, the serum contains at least 3 AU/ml of *C. perfringens* epsilon antitoxin, and the product is satisfactory.

5.2.3 The product is considered unsatisfactory if the serum pool from at least 7 rabbits contains less than 2 epsilon AU/ml. (If any mice inoculated with the 1:2 serum dilution and 10 L_o doses of standard epsilon toxin die, the product is considered to contain less than 2 AU/ml.)

6. Report of test results

Report results of the test(s) as described by standard Section operating procedures.

7. References

7.1 Code of Federal Regulations, Title 9, Part 113.112, U.S. Government Printing Office, Washington, DC, 2005.

7.2 History of toxin: *C. perfringens* type D epsilon culture CN3688, used to produce IRP 450, was obtained from Coopers Animal Health, Inc., 1201 Douglas Avenue, Kansas City, Kansas 66103-1438, on January 5, 1976. The number of passages is unknown.

7.3 History of antitoxin: *C. perfringens* type D epsilon antitoxin (IRP 249) was produced in 1981 at the Center for Veterinary Biologics (CVB) (then part of the National Veterinary Services Laboratories [NVSL]), Ames, Iowa. The antitoxin is of equine origin.

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8. Summary of revisions

This document was revised to clarify the practices currently in use at the Center for Veterinary Biologics and to provide additional detail. While no significant changes were made that impact the outcome of the test, the following changes were made to the document:

- Humane endpoint language has been added.
- Dilution/holding vessel sizes have been added for clarification.
- The contact person has been changed to Janet M. Wilson.

Version .02

- Incorporated the use of IRP 450.
- **4.4.1.2** Corrected a typographical error.

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